STRUCTURE OF LIPID TUBULES FORMED FROM A POLYMERIZABLE LECITHIN

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ABSTRACT We have studied tubules formed from a polymerizable lipid in aqueous dispersion using freeze-fracture replication and transmission electron microscopy. The polymerizable diacetylenic lecithin 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine converts from liposomes to hollow cylinders, which we call tubules, on cooling through its chain melting phase transition temperature. These tubules differ substantially from cochleate cylinders formed by phosphatidylserines on binding of calcium. The tubules have diameters that range from 0.3 to 1 μm and lengths of up to hundreds of micrometers depending on conditions of formation. The thickness of the walls varies from as few as two bilayers to tens of bilayers in some longer tubules. Their surfaces may be either smooth, gently rippled, or with spiral steps depending on sample preparation conditions, including whether the lipids have been polymerized. The spiral steps may reflect the growth of the tubules by rolling up of flattened liposomes.

INTRODUCTION

Since the earliest studies on phospholipids in aqueous dispersion (Bangham et al., 1965), pure lecithins have always been found in liposomal form, with an aqueous space contained by single or multiple continuous bilayers. This is true even for synthetic lecithins with complex thermal properties, such as dipalmitoyl phosphatidylcholine, which has at least has three phase transitions (Chen et al., 1980). The phase transitions may change the bilayer spacings (Inoki and Mitsui, 1978), and also the surface areas of the liposomes (Yager et al., 1982; Evans and Kwok, 1982), but the topology of the liposomes remains unchanged. Even though sonicated small unilamellar lecithin vesicles below their phase transition temperature are considered unstable because they fuse to slightly larger unilamellar vesicles (Suurkuusk et al., 1976; Gaber and Sheridan, 1982; Chang et al., 1982), they are still never seen in nonspheroidal form. We report here a detailed analysis of a lecithin-water system that, as we recently discovered (Yager and Schoen, 1984), forms hollow tubes in the low temperature phase.

The lecithin, 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{23}PC$), contains a diacetylenic group halfway down each of its 23-carbon hydrocarbon chains. Diacetylenes polymerize to rigid linear polyenes via a 1–4 addition reaction if irradiated with 254 nm light or more energetic radiation, such as gamma rays or high energy electrons (Wegner, 1969). The reaction only proceeds when the monomers are properly aligned, as in crystals, and not in the melt (Baughman and Chance, 1978). The lipid $DC_{23}PC$ can be converted to a dark red polymer, but only when it is below its phase transition temperature (T_m) of 40°C (Johnston et al., 1980; O'Brien et al., 1981; Lopez et al., 1982; Leaver et al., 1983). We have been studying the chemistry and structure of $DC_{23}PC$, including monitoring the conformation of polymerizable hydrocarbon chains before and after polymerization using infrared and Raman spectroscopy (Schoen and Yager, 1985; Schoen et al., 1985).

On examining the phase transition of a monomeric polymerizable diacetylenic lecithin under the optical microscope we found, to our great surprise, that the quite normal liposomes formed by gentle dispersion of the lipid above its phase transition temperature (T_m) became unstable and appeared to disintegrate on cooling through T_m (Yager and Schoen, 1984). If the monomeric lipid was cooled rapidly to below 30°C, the liposomes violently broke into small shards, but when cooled slowly to 37° or 38°C, which is within the rather broad melting transition of the compound, the liposomes converted quantitatively to hollow tubes over a period of a minute. These tubules were between 0.3 and 1 μm in diameter, with fairly thin walls, and ranged in length from a few to hundreds of micrometers. After polymerization, the tubules no longer converted to liposomal form when heated, but did exhibit thermochromism, indicating temperature effects on the conformation of the chromophoric polymer.

While phosphatidylcholines have been considered topologically inert, other classes of lipids, such as phosphatidylethanolamines, phosphatidylglycerol, cardiolipidin, and other charged lipids can convert to nonlamellar phases, such as the inverted hexagonal (H_{II}) phase (Cullis and de Kruijff, 1979), or in the case of phosphatidylserines in the presence of Ca^{2+}, a rolled-up lamellar phase dubbed cochleate cylinders (Papahadjopoulos et al., 1975). Struc-

observed for a diacetylenic lecithin with 20-carbon long fatty acyl chains, and were assumed to be similar to cochleate cylinders (Leaver et al., 1983). Tubules and cochleates are superficially similar, but our initial electron microscopic studies on $DC_{23}PC$ indicated that the tubules were somewhat different from cochleate cylinders in being open ended, and often consisting of only a few bilayers (Yager and Schoen, 1984). Cochleate cylinders are similar in diameter, although they are not nearly so long as tubules, and consist of very tightly wrapped multibilayers with little or no internal aqueous space (Papahadjopoulos et al., 1975). Recently a tubular lipid structure has been observed to form from an amino acid–based surfactant (Nakashima et al., 1985) that is more similar in appearance to the tubules described here than is the cochleate cylinder. To differentiate the tubules from cochleate cylinders and further refine our understanding of the tubule structure, we have extended our preliminary studies to include freeze fracture of samples in several states, and transmission electron microscopy of air-dried unstained tubules. We have found that the various images observed suggest not one but a number of different models for tubule formation.

MATERIALS AND METHODS

The lipid 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine was synthesized in our laboratory by previously published methods (Johnston et al., 1980) and purified to a single spot by thin layer chromatography in a chloroform/methanol/water system. It was found that some impurities in the lipid could prevent the formation of tubules, but that completely pure lipids always formed tubules if properly handled. To make tubules, the lipid was dried from chloroform under dry nitrogen in glass conical vials in a Meyer N-Evap evaporator (Organomation Associates, Inc., South Berlin, MA). The partially dried lipid was placed in high vacuum for 12 h to allow remaining solvent to evaporate. The lipid was hydrated at room temperature using distilled water and placed in an incubator at 60°C. After 2 h of incubation the vials were gently shaken and the temperature reduced to 38°C at a rate of ~0.4°C/min. Once the temperature had equilibrated, an hour was needed to allow the tubules to settle out of solution. Subsequently they were stored at room temperature or at 4°C. When required, tubes were polymerized by exposure to 254 nm light in a Rayonet reactor (Southern New England Ultraviolet Company, Hamden, CT) until a dark red color was observed.

Optical microscopy was performed with a Leitz Ortholux I (E. Leitz, Inc., Rockleigh, NJ) with darkfield illumination at room temperature. For transmission electron microscopy, tubules in distilled water were pipetted onto carbon-coated grids, air dried, and observed directly. All electron micrographs were taken on a transmission electron microscope (model EM200; Philips Electronics Instruments, Inc., Mahwah, NJ). For freeze fracture the cryoprotectant glycerol was added to samples to a final concentration of 10% by weight. Specimens were then transferred to Balzers (Hudson, NH) copper specimen plates, equilibrated at room temperature, and quickly frozen by plunging into melting nitrogen. They were then transferred to a Balzers 360 or BAF 400D freeze fracture device, fractured, in some cases briefly etched, and then replicated at −100°C and 10^{-6} Torr. Replicas, which were made with 2 nm Pt-C film and 20 nm of carbon, were floated off onto distilled water and transferred to sodium hypochlorite for 2 h, rinsed in bidistilled water, and cleaned on 20% ethanol for 1 h. They were then picked up on Butvar B-98 (Monsanto Corp., St. Louis, MO) coated grids and examined. Unless otherwise indicated, electron micrographs of replicas have been printed with dark original plate from the microscope. This allows a more natural interpretation of structures that have extreme relief and long shadows, as is often the case for the tubules. The samples seen in freeze fracture micrographs here were all originally hydrated in 10 mM EDTA, but no differences between these samples and those prepared in distilled water have been observed.

RESULTS

When the conditions are optimal, quantitative conversion to tubules is possible, as can be seen in Fig. 1, an optical micrograph of monomeric tubules viewed with darkfield optics. At this magnification the walls of the tubules can just be resolved, and at higher powers it can clearly be seen that their ends are open (Yager and Schoen, 1984). This sample is typical for preparation in distilled water under the conditions described in the Materials and Methods section.

The lipid vesicles from which tubules are to be formed must be larger than a micrometer or so in diameter, otherwise shards rather than tubules were formed no matter what the temperature. Conditions that generate excessive numbers of small vesicles include sonication or even gentler forms of agitation, such as vortexing; contamination from decomposition products formed by long-term storage of the lipid in chloroform also seems to promote the formation of small vesicles. Freeze-fracture electron microscopy of this nontubular material as seen in Fig. 2 reveals that it consists of some small liposomes and shards of curved lipid bilayers that appear to be pieces of incompletely formed tubules. Apparently, the formation of complete tubules requires the presence of large liposomes or the aggregation and/or fusion of small liposomes; these processes become impossible if the lipids are supercooled or

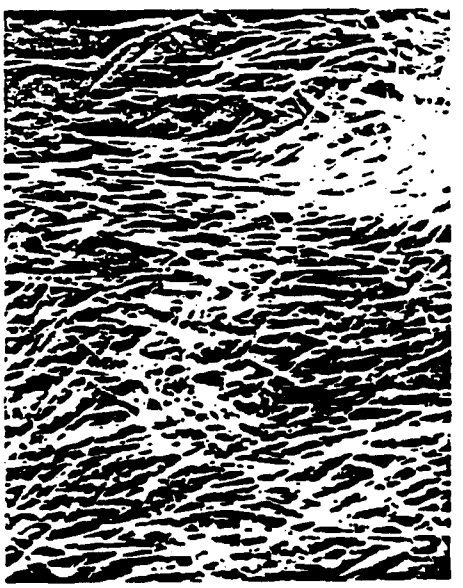

FIGURE 1 Optical micrograph of $DC_{23}PC$ tubules in distilled water taken with darkfield illumination. The walls of the tubules can just be resolved. Scale bar, 25 μm.

FIGURE 2 Freeze fracture micrograph of lipid fragments too small to appear tubular by optical microscopy. Some of these appear to be partially formed tubules. Scale bar, 0.5 μm.

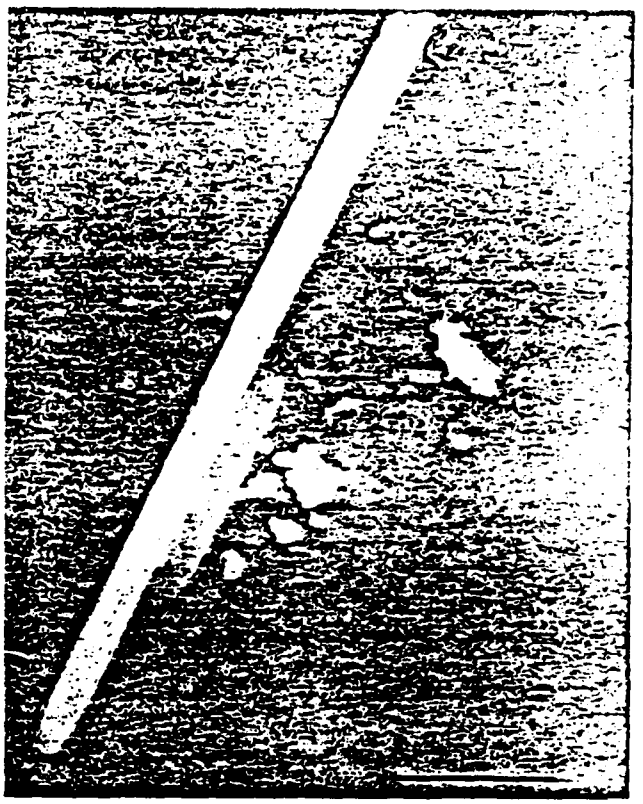

FIGURE 3 Transmission electron micrograph of unstained monomeric tubules. Such samples often show more wrinkling of the tubule walls than is apparent here. The smaller tubule appears to share a sheet of lipid with the larger, as the wrapping lines are continuous from one to the other. Scale bar, 5 μm.

dispersed too finely. The basic cylindrical structure of the tubules and the approximate radius of curvature is reflected in the shards.

When complete tubules in distilled water are air dried onto a carbon film and observed under the electron microscope without staining, the lipids scatter enough electrons to produce images such as that shown in Fig. 3. Stereo pairs made from this type of sample show that drying flattens the tubules onto the film surface, collapsing the internal hollow space. The electron dosage used to observe the sample and take such micrographs with the EM200 was quite high, and considering the sensitivity of diacetylenes to polymerization by a wide range of radiation, it is likely that the lipid in the beam had been polymerized by the electrons by the time the image was made. This assumes that the hydrocarbon chains were still ordered after drying and then being heated by the electrons. It is also possible that the electron beam had decomposed the lipid to amorphous carbon. Nonetheless it is unlikely that a polymerization or decomposition artifact could generate the startlingly regular wrapping of discrete layers as seen in Fig. 3. The apparent spiral wrapping of a strip of lipid ~2 μm wide, to make up the walls of the structure, has been seen consistently in monomeric tubules that are thin enough to allow resolution of their internal structure. A good model is a flattened paper soda straw. It is not apparent from transmission images exactly how thick is the piece that wraps around the tubule axis, although it is certainly as thick as a monolayer and perhaps as thick as two or more bilayers. The actual number of bilayers should provide a clue to the method of formation of the tubules.

We have previously observed irregular helical striations on the surface of dry, monomeric tubules subjected to electron bombardment (Yager and Schoen, 1984) that we have attributed to a change in the packing of the lipid hydrocarbon chains during polymerization. This interpretation is supported by the absence of such fibrous structures in micrographs of tubules that have been at least partially polymerized before electron microscopy and then stored in water for at least several days. Such tubules appear to have been annealed, in that the regular helical banding pattern is often completely absent, leaving a featureless surface, as seen in Fig. 4. This sample was formed from a lipid dispersion that was frozen in water, warmed to 45°C, then slowly cooled without any agitation to 38°C. The lack of agitation may have resulted in larger

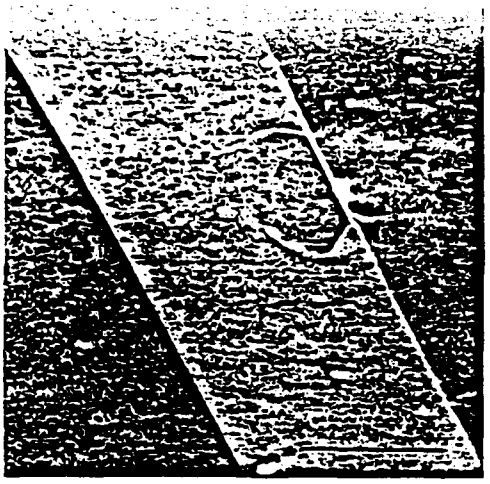

FIGURE 4 Transmission electron micrograph of tubule that was polymerized and stored for over a month at 4°C before observation. It was prepared in the same manner as the sample in Fig. 3. Scale bar, 0.5 μm.

liposomes, and hence tubules made from larger pieces of bilayer, or the storage period may have allowed the energetically unstable overlapping edges present in newly formed tubules to fuse.

The flattening of tubules that occurs on drying does not occur when the samples are freeze fractured, allowing visualization of their true cylindrical cross section. To reduce ice crystal artifacts the samples used here were frozen after addition of 10% glycerol. While we have consistently found that high glycerol concentrations seem to reduce the tightness of lamellar wrapping of the tubule walls, it is still not known how glycerol affects the structure of those bilayers. For aqueous dispersions of dipalmitoyl phosphatidylcholine, for example, high concentrations of glycerol cause a collapse of the normal bilayer structure into one in which the hydrocarbon chains are interdigitated (McDaniel et al., 1983), so a certain amount of caution is advisable in interpreting samples prepared in glycerol. We have, however, prepared tubules in glycerol concentrations as high as 90% with little change in tubule morphology, as observed by optical microscopy (Yager et al., manuscript in preparation).

That tubules are in fact filled primarily with water can be seen from the image in Fig. 5 of two tubules fractured at a shallow angle near their ends. In one of these tubules there are a few bilayers folded into the tubule lumen, although optical micrographs at high magnification usually give the impression that the tubes are completely hollow. In these samples the glycerol was added shortly before freezing, so that some delamination may have occurred. If the tubules are only formed by rolling up of flattened liposomes, one would expect there always to be even numbers of bilayers making up the tubule walls. One can count bilayers from the center of the tubules outward in Fig. 5, giving three bilayers in one case and five in the other. This implies that some unflattened liposomes may

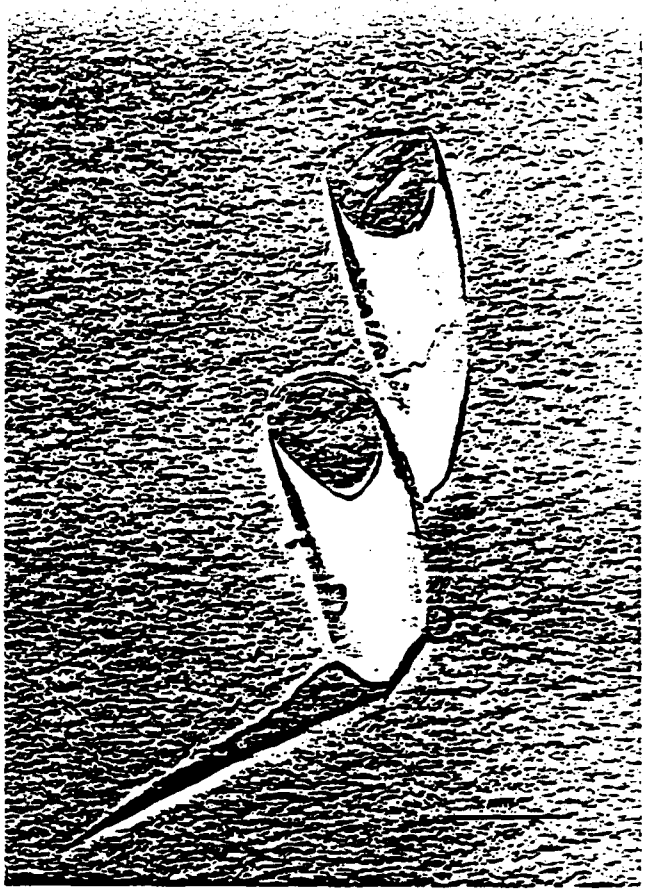

FIGURE 5 Freeze fracture replica of the concave ends of two tubules. Because of their slightly disordered interiors one can count three bilayers making up the wall of one, and five in the other. The apparent noncylindrical cross section of one of the tubules is not typical and may be the end of the tubule. Scale bar, 0.5 μm.

become trapped in the tubule interior as other flattened liposomes wrap around it.

The wrapping of bilayers is often not completely regular, as can be seen in the tubule in Fig. 6. The apparent taper to the tubule is probably caused by the slight angle between the tubule and the fracture plane. We have so far only utilized the natural chiral phosphatidylcholine head group in our $DC_{21}PC$, which always produces tubules with a right-handed helical wrapping. Note the thinness of the walls of the tubules and the openness of the lumen in Fig. 7. The aqueous interior in this tubule is entirely uninterrupted, and the walls are clearly made up of very few bilayers. In other micrographs not shown here, we have seen cross sections of the ends of tubes that were clearly no more than two bilayers in thickness. Close examination of Fig. 7 shows what appear to be flattened vesicles making up at least part of the walls. These may even be contiguous with the large liposome floating near the tubule, or to the smaller one apparently attached to the tubule.

A similar situation can be seen in Fig. 8, but in this case it is quite clear that there are one or two flattened

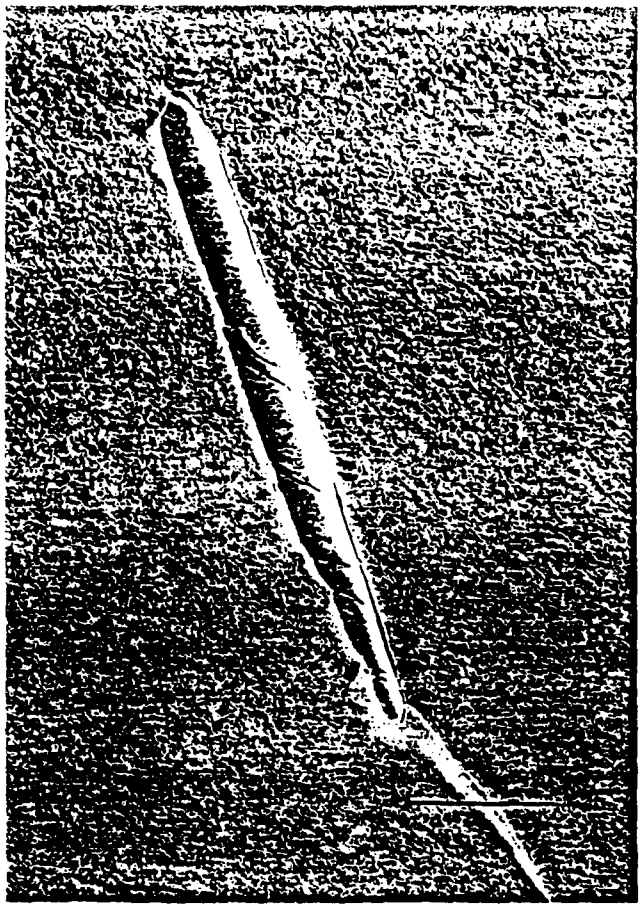

FIGURE 6 A concave freeze fracture replica of a tubule with stepped helical wrapping. The wrapping forms a right-handed helix, as it does in all tubules formed from this isomer of the lipid. Scale bar, 1 μm.

liposomes of varying width that wind around the tubule to form a helical sheath. The part of the tubule that can be seen in Fig. 8 has a completely smooth cylindrical core (which appears to be continuous with one of the cross-fractured liposomes at the tubule's end) around which the liposome is wrapping. As mentioned above such a structure would be expected to have a wall containing an odd number of bilayers, whereas if tubules were made entirely from flattened vesicles they would always have walls with even numbers of bilayers. The presence of the smooth shaft in the presence of wrapped liposomes again suggests that tubules can be made up of two types of bilayers—those added by wrapping flattened liposomes, and those made tubular as they were trapped by other liposomes wrapping around them.

Aside from the diameter of the tubules, the most regular feature we have observed associated with them is a shallow rippling of the tubule wall frequently observed in polymerized samples. This ripple, as seen in Fig. 9, makes an angle of ~30° to the tubule axis, and has a period of 100 nm along the tubule. The amplitude of the ripple is extremely low, and only fortuitous alignment of the ripple perpendicular to the shadowing direction allows it to be seen. Along with the possible loss of the overlapping segments, as seen in Fig. 4, the production of this rippling in the surface is the only polymerization-induced morphological change in the tubules that we have observed by freeze fracture. It is interesting that the fibrous striations observed in monomeric tubules in unstained transmission specimens are also arranged at ~30° to the tubule axis. We have previously speculated as to the probable helical alignment of the polymer chains relative to the tubule axis (Yager and Schoen, 1984). As it is unlikely that the polymer chain spacing is identical to the spacing of the monomers along the chain propagation direction, formation of the polymer is likely to cause strain and deformation in the tubule structure. While the relation of this pattern to the underlying polymer chains is not proven since the crystal structure of the walls of the tubules is as yet unsolved, this rippling does provide strong circumstantial evidence for regular helical wrapping of the polymer.

DISCUSSION

There is no obvious physical reason for liposomes, cooled below the melting point of their constitutent lipids, to form tubes with a particular, narrowly defined diameter, but it is clear that the crystalline lipids making up the tubule walls must favor a finite degree of curvature in one direction, and no curvature in the other, to produce the tubule structure. Chirality is necessary but not sufficient to induce helical structures with a consistent handedness. We originally believed that the kink in the hydrocarbon chain produced by the diacetylene group was responsible for the unusual behavior of $DC_{23}PC$, as other lecithins with long hydrocarbon chains show no inclination to form tubules (Schoen and Yager, 1985). Some similar diacetylenic phospholipids with slight changes in hydrocarbon chain lengths do form tubules, and others do not, but we have not yet established a pattern.

The kink theory was severely tested by the aforementioned paper from Kunitake's laboratory (Nakashima et al., 1985) that reports the formation of nearly identical tubulelike structures from a totally dissimilar lipid. Their tubule former is based on glutamic acid, with two *n*-dodecyl chains attached to the carboxyl groups, and a third long chain with a quaternary ammonium terminal group attached via a peptide linkage. Below the phase transition temperature of this lipid, liposomes convert to long regular helical structures that slowly convert over a period of weeks to straight tubes with dimensions very similar to those produced by $DC_{23}PC$. The glutamate-based lipid is chiral, and the handedness of the helices depends on the chirality of the amino acid, so that racemic mixtures produce no tubules. It appears that the tubule is a thermodynamically stable structure that is common to several lipid systems. The extremely slow conversion to the final tubules in the glutamic acid lipid system suggests that the formation

FIGURE 7 A convex freeze-fracture replica of a tubule with thin walls and an adjacent multilamellar liposome. Note the thin walls and adherent liposomes, including an extremely flattened one. This micrograph is printed with white shadows to improve contrast. Scale bar, 0.5 μm.

mechanism in that system may be unrelated to the $DC_{21}PC$ mechanism.

Several mechanisms suggest themselves for the formation of the tubules from the parent liposomes, but these mechanisms are so unusual that they defy description with standard scientific jargon. Single, large liposomes may somehow deflate and curl themselves up like window shades; alternatively, pairs or even bundles of deflated vesicles may wrap around each other like cigar leaves. The twisting of elongated vesicles around each other could result in the soda straw structure of Fig. 3. These would yield tubules with walls containing even numbers of bilayers. If helical liposomes, as formed in mixed cardiolipin-phosphatidylcholine systems on addition of Ca^{2+} (Lin et al., 1982), were to collapse appropriately, tubules might result. A somewhat different mechanism would involve a central vesicle being captured and entwined by deflated vesicles. Part of the central vesicle could bulge out from the end of the tubule as is suggested by Fig. 8. Such tubules would have an odd number of bilayers in their walls if the central captured vesicles were made of odd numbers of bilayers. Smaller vesicles, still spherical in shape, or perhaps partially deflated, could nestle against the side of an already formed tubule, waiting for a sufficiently energetic stimulus to finish the wrapping process. Examples of this can be inferred from Figs. 5, 7, and 8.

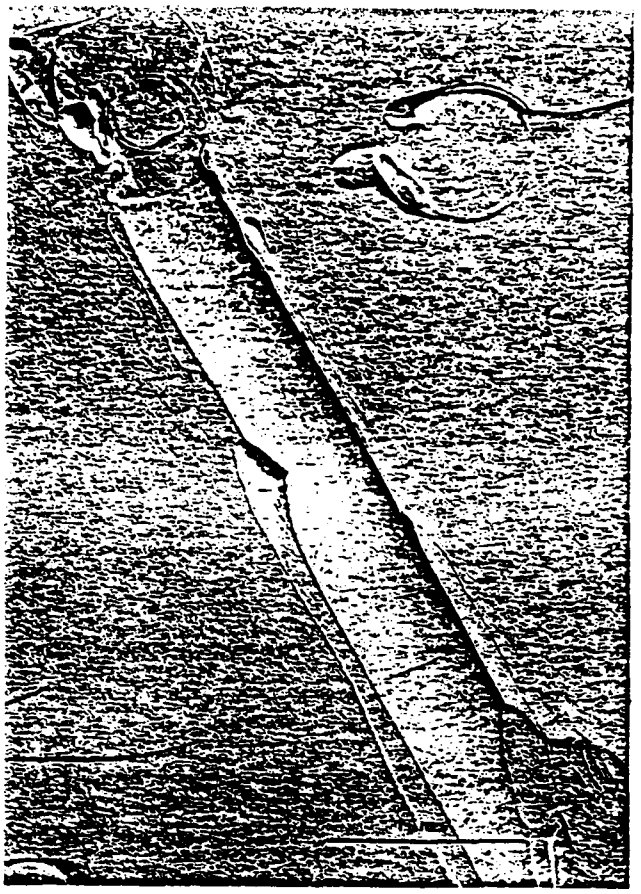

FIGURE 8 Freeze-fracture micrograph of tubule with flattened liposome wrapping around it and liposome contiguous with the smooth core. Note that the replica is concave. Scale bar, 1 μm.

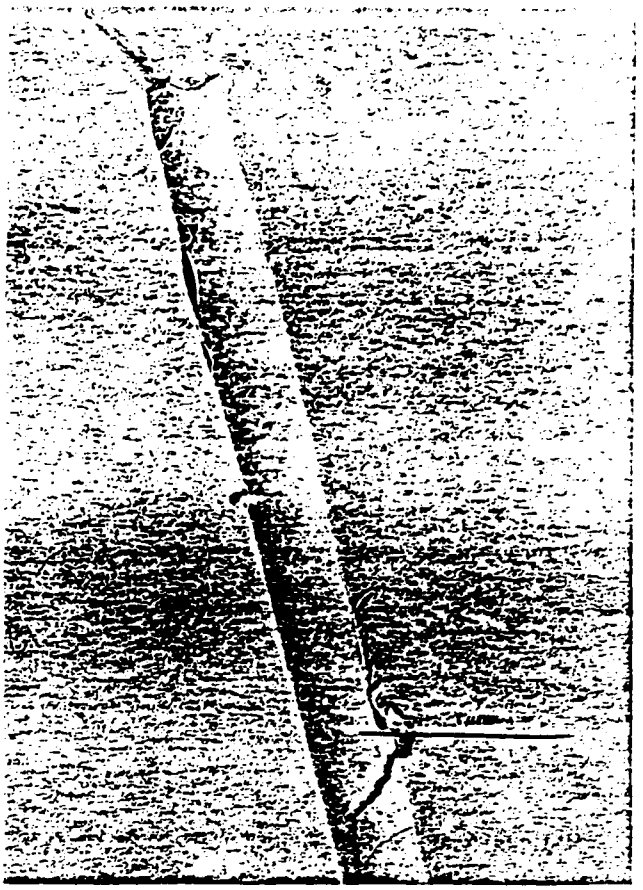

FIGURE 9 Freeze fracture micrograph of the convex surface of a tubule demonstrating the helical pattern of shallow ridges often seen wrapping around the axis of polymerized samples. Flattened liposomes are also present. Scale bar, 1 μm.

A third mechanism is suggested by the soda straw structure in Fig. 3. In the violent deflation of a vesicle, a single strip of bilayer could tear loose and wrap around itself or around an already formed tubule. This latter mechanism has the unfavorable aspect of at least temporarily exposing, at the edge of the strip, the hydrophobic region of the bilayer to water. Wrapping of such a strip followed by annealing could produce tube walls a single bilayer thick. A compromise mechanism involves elongation of the tubule by pinching off a piece of liposome surface that would flatten into a double bilayer strip a micrometer wide. The strip would then wrap around a rotating tubule. In this mechanism no hydrocarbon edges would be exposed until so much material is peeled off the liposome that it ruptures. Such a rupture may have been seen in Ca^{2+}-induced fusion of phosphatidylserine vesicles (Rand et al., 1985). Observation of the formation process under the optical microscope suggests that tubules could be produced by this latter method since the tubules appear to rotate while dragging in material from surrounding liposomes. We have recently been able to trap and observe the intermediates in the conversion process by a freeze-fracture microscopy (Yager et al., submitted for publication), and the results strongly support the last described mechanism. We have also recently formed tubules by an entirely different mechanism (Georger et al., submitted for publication), producing intermediates nearly identical to the helical structures seen in Kunitake's glutamate-based tubules.

There clearly are differences between the tubules and cochleate cylinders that merit establishing the tubules as a unique class of lipid structure, but there are probably similar processes occurring during the formation of each. The conversions to cochleate cylinders from phosphatidylserine liposomes and to tubules from liposomes of $DC_{11}PC$ appear to be driven by the phase transition to the gel state of the lipid hydrocarbon chains, induced isothermally by binding of Ca^{2+} in one case and by cooling in the other. Also, when the low temperature forms are reheated, liposomes are reformed from them. When the warm liposomes are less than a certain size tubules will not form at all, and the cochleate cylinders will only form after fusion of small liposomes to much larger ones (Wilshut et al., 1985). Given these superficial similarities, one might expect similar molecular mechanisms as well. Dehydration of headgroups is known to play a vital role in allowing the close

cylinder formation (Portis et al., 1979), and it may be that similar dehydration occurs when $DC_{21}PC$ liposomes contact as a first step in the formation of tubules. Binding Ca^{2+} drives the conversion of the phosphatidylserine bilayer to the gel phase, and the resultant close apposition of bilayers (as reflected by the extremely short bilayer repeat of 5.4 nm) in the cochleate cylinders (Papahadjopoulos et al., 1975). We have not yet measured the bilayer repeat in the tubules in distilled water, but we have regularly seen a tendency for some bilayers to be separated by large distances in tubules formed in varying amounts of glycerol. Clearly close bilayer apposition is not so important a phenomenon for tubules as it is for cochleates.

Several uses for such structures as the tubules suggest themselves. Much of the interest in the properties of cochleate cylinders derives from the fact that when the Ca^{2+} is chelated away from the phosphatidylserine, large unilamellar liposomes are formed that efficiently trap the surrounding aqueous medium (Papahadjopoulos et al., 1975). If the $DC_{21}PC$ tubules are not polymerized, they too will convert to liposomes on heating, and for this reason may also prove useful for entrapping solutes. If present at high concentration, these liposomes would presumably be stable as long as the temperature was kept above ~39°C, but would expel their contents on cooling and reconversion to tubules. Possible uses for selective drug delivery to cool tissues might be envisioned.

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